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(54) Title: PEPTIDE BINDING ASSAYS WITH MHC ANTIGENS

(57) Abstract

Rapid, efficient, accurate assays are provided for measuring affinity of small molecular weight organic molecules and peptides to an MHC glycoprotein. The method employs a competition between a detectable agonist and the candidate of interest for an MHC glycoprotein in solution or coupled to solid support, followed by detection of complex formation of the MHC glycoprotein and agonist. The MHC glycoprotein may be preloaded with a second agonist to optimize the assay. Alternatively, the method employs the displacement of a preloaded, labeled peptide from the MHC glycoprotein by the candidate of interest. The level of agonist in the complex may be determined by the effect of the complex on sensitized T-cell proliferation.

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PEPTIDE BINDING ASSAYS WITH MHC ANTIGENS

Technical Field

The invention is related to methods to determine the ability of candidate moieties to bind to specific major histocompatibility complex glycoproteins. Successful candidates are useful as therapeutics in conditions mediated by particular MHC glycoproteins.

Background of the Invention

Because the immune system is such an essential part of the well-being of mammalian species, there has been intense research in attempting to understand how the immune system works. Only recently was it determined that the major histocompatibility complex (MHC) glycoproteins play an essential role in B-lymphocyte and T-lymphocyte responses. The MHC glycoproteins are divided into two types, Class I and Class II, where each of these classes appears to play a substantially different role. In addition, the structure of the Class I and Class II glycoproteins is different. Despite the differences, in each case it is found that an antigen to which the immune system will respond is degraded intracellularly and a fragment of the antigen is expressed on the cell surface associated with an MHC glycoprotein. Each MHC glycoprotein has polymorphic

regions, which are associated with a groove, and have some degree of specificity as to the peptide which binds in the groove. Thus, each MHC glycoprotein has only a specific repertoire of relatively low molecular weight moieties to which it is capable of binding. It is the identification of this repertoire of low molecular weight moieties to which the invention described below is directed.

The MHC glycoprotein-peptide complex is presented to a T-cell receptor which specifically recognizes the fragment in conjunction with the MHC glycoprotein to which it is bound. The T-cell becomes stimulated and secretes lymphokines, with resulting expansion of the lymphocytes.

Since these fragments are critical to the activation of lymphocytes, peptides or small molecular weight organic molecules may be devised or discovered which will play a role in enhancing or diminishing the activation of specific lymphocytes. In this way, B- and T-lymphocyte activation may be controlled to enhance or suppress a particular immune response. It is therefore of substantial interest to be able to identify successful candidate moieties which activate or inhibit lymphocyte response, where these identified moieties may find use in the treatment of autoimmune disease, infection, allergies, and the like. Thus, determination of moieties capable of binding a specific MHC glycoprotein of interest provides potential therapeutic compounds for use in conditions mediated by presentation of antigen moieties by the relevant MHC glycoprotein.

Ordinary competitive binding assays do not appear to be appropriate for identifying suitable candidate moieties. Unlike more conventional ligand/receptor binding, the rate of formation of the moiety/MHC glycoprotein complex is very slow, but the

resulting complex is stable. As a consequence, MHC glycoproteins obtained from native materials are complexed with a heterogeneous array of endogenous peptides and are thus prevented from binding candidate alternate moieties, except at a very slow and variable rate. It is estimated that only about 1% of isolated MHC glycoproteins are in an "open form" that is capable of directly binding alternate moieties; about 99% of the preparation is already complexed with endogenous peptide.

Thus, there is a need to devise assays for successful ligand binding which allow screening of large numbers of candidate moieties with respect to a particular MHC glycoprotein in a rapid and efficient manner. The invention provides several protocols to achieve this result.

Relevant Literature

The following references are believed to be relevant. Babbitt et al. (1985) "The Binding of Immunogenic Peptides to Ia Histocompatibility Molecules" Nature 317:359-61; Buus (1986) "Isolation and Characterization of Antigen-Ia Complexes in T-cell Recognition" Cell 47:1071-77; Watts and McConnell (1986) "High Affinity Fluorescent Peptide Binding to I-A^d in Lipid Membranes" Proc Natl Acad Sci USA 83:9660-4; Busch et al. (1990) "Degenerate Binding of Immunogenic Peptides to HLA-DR Proteins on B-cell Surfaces" Int Immunol 2:443-1; Roche and Cresswell (1990) "High Affinity Binding of an Influenza Hemagglutinin-derived Peptide to Purified HLA-DR" J Immunol 144:1849-56; Chen and Parham (1989) "Direct Binding of Influenza Peptides to Class I HLA Molecules" Nature 337:743-5; and Bouillot et al. (1989) "Physical Association Between MHC Class I Molecules and Immunogenic Peptides" Nature 339:473-5.

Disclosure of the Invention

In one protocol provided by the invention, dispersed soluble MHC glycoproteins are treated with a detectable agonist in the presence of a competitor candidate moiety under conditions wherein the agonist is known to form a complex with the MHC glycoprotein. The resulting complex is separated from the reaction mixture, and the effect of the candidate moiety on the agonist included in the complex is determined.

In a second protocol, especially appropriate for instances where purified MHC glycoprotein is not readily available, MHC glycoprotein is captured on an assay plate which plate is, for example, derivatized to anti-MHC glycoprotein antibody or other reagent with affinity for the MHC glycoprotein and used in place of the soluble MHC in the competitive assay. Again, the effect of the candidate moiety on the binding of the immobilized MHC to detectable agonist is determined.

In an additional protocol, isolated MHC glycoprotein is preloaded with a homogeneous peptide preparation to provide, therefore, a homogeneous population of already-coupled MHC glycoprotein. As the dissociation of the preloaded peptide is the rate-determining step in the association of alternate moieties, this approach permits control of the reaction rate to assure uniform competition between the detectable agonist and the candidate. In addition, dilution of the performed MHC complex results in rapid dissociation of the preloaded peptide, thus creating an "empty pocket" (i.e., an available domain or binding site) for binding to the agonist or candidate. The homogeneous, preloaded peptide is preferably chosen to be comparatively readily released by the MHC glycoprotein, thus shortening the time of the assay.

In another protocol, isolated MHC glycoprotein is preloaded with a labeled peptide whose rate of dissociation from the MHC glycoprotein is known to be influenced by the presence of other peptide. The ability of test compounds to accelerate the dissociation or displacement of the labeled agonist from the MHC glycoprotein is determined.

In addition, a useful means to detect the formation of complex with the agonist and/or candidate moiety is to determine the effect of the complex on T-cell stimulation.

Thus, in one aspect, the invention is directed to a method to determine the affinity of a test compound to an MHC glycoprotein, which method comprises combining in a reaction mixture cell-free dispersed MHC glycoprotein, a soluble agonist capable of binding to said MHC glycoprotein to form a complex and capable of being detected when in said complex, and said test compound, under conditions wherein the test compound and the agonist compete for binding to the MHC glycoprotein; separating MHC glycoprotein-bound agonist from unbound agonist; and detecting the amount of agonist bound in the complex as a function of the concentration of test compound in the reaction mixture.

In a second aspect, the invention is directed to a method to determine the affinity of a test compound for a specific MHC glycoprotein, which method comprises treating a solid support to which the MHC glycoprotein is coupled with a reaction mixture containing a soluble agonist capable of binding to the MHC glycoprotein to form a complex and capable of being detected when in said complex and said test compound, under conditions wherein the test compound and said agonist compete for binding to the MHC glycoprotein; removing said reaction mixture from the solid support; and detecting the amount of agonist

bound to the solid support as a function of the concentration of the test compound in the reaction mixture.

In a third aspect, the invention is directed to a method for determining the affinity of a test compound to an MHC glycoprotein, which method comprises treating an MHC glycoprotein preloaded with a first agonist with reaction mixture containing the test compound and a second agonist capable of binding to said MHC glycoprotein to form a complex and capable of being detected when in said complex under conditions wherein the test compound and second agonist compete for binding to said MHC glycoprotein; wherein said MHC glycoprotein is preloaded with a preloading agonist and diluted prior to the addition of the reaction mixture; and detecting the amount of second agonist bound in the complex as a function of the concentration of test compound in the reaction mixture.

In a fourth aspect (a dissociation rate assay), the invention is directed to a method for determining the affinity of a test compound to an MHC glycoprotein, which method comprises combining in a reaction mixture the test compound and said MHC glycoprotein which has been preloaded with a labeled agonist, whose dissociation rate has been shown to be affected by the presence of other peptides and comparing the dissociation rate of the preloaded complexes in the presence and absence of the test compound to detect any accelerated off rate by the test compound.

In a fifth aspect, the invention is directed to a method to detect the presence of a moiety in an MHC glycoprotein complex which method comprises contacting said complex with a culture of T-cells primed with said moiety and detecting the presence, absence or amount of proliferation of said T-cells.

Brief Description of the Drawings

Figure 1 shows a typical inhibition curve wherein unlabeled HA 307-319 competes with labeled HA 307-319 for binding to DR4Dw4.

Figure 2 shows a binding curve for labeled HA 307-319 using DR4Dw4 captured from a crude lysate by antibody-coated microtiter plates.

Figures 3A, 3B and 3C show "off rates" determined for various test peptides after preloading onto DR4Dw4 MHC glycoprotein.

Figures 4A and 4B show a binding curve and an inhibition curve, respectively, for RMBP 90-102 peptide with respect to preloaded and control MHC glycoprotein.

Figures 5A and 5B show dissociation curves for, respectively, biotinylated RMBP 90-102 and biotinylated HSP 3-14 in the absence and presence of HA 307-319.

Figure 6 shows a displacement curve of biotinylated HSP 3-14 by RMBP 90-102.

Figure 7 shows a binding curve for HA 307-319 peptide using sensitized T-cell proliferation as an assay for bound peptide.

Modes of Carrying Out the Invention

Methods and compositions are provided for determining the binding affinity of a candidate moiety for a specific MHC glycoprotein using competition between detectable agonist and the candidate of interest using several efficient assay strategies.

In all of the strategies of the invention, except the dissociation rate assay, competition is effected between a detectable agonist and the candidate. The effect of at least one concentration, and preferably varying concentrations, of the candidate moiety on binding of the detectable agonist is then determined.

In the dissociation rate assay, the effect of at least one concentration, and preferably varying concentrations of the candidate moiety, on the dissociation rate of the preloaded MHC/labeled agonist complex is determined.

By detectable "agonist" is meant a peptide or other low molecular weight compound known to be capable of binding to the specific MHC glycoprotein being tested. Useful agonists for DR alleles, for example, are peptides derived from hemagglutinin such as that represented by positions 307-319 (HA 307-319) (Int Immunol (1990) 2:443). Other useful agonists that have been found to bind MHC glycoprotein products of DR alleles include, but are not limited to, HADP 3.2, RRFAAAYAARAAA; HADP 3.6, RRFAAQYAARAAA; RMBP 90-102, HFFKNIVTPRTPA; HSP 3-14 (R5 K13), RVRRGLTVAVKG; HSP 3-14 (K5 K13), RVKRGLTVAVKG; HSP 3-14 (K5 A13), RVKRGLTVAVAG and shortened versions thereof. Useful agonists may include the peptides shown above and equivalents in which the amino and carboxyl charges eliminated. Some agonists are allele specific such as pertussis toxin 31-43, ragweed 3 50-62 and flu matrix 18-30.

The agonist is generally labeled in a manner so as to permit its convenient detection when complexed to MHC. However, if detection is effected in a manner which is dependent on the nature only of the agonist, as described with respect to the T-cell proliferation assay for determination of complexed peptide, no extraneous labeling is needed. Thus, the agonist need only be detectable when in the complex.

However, if the agonist is detected by means of a label, the label may be in various forms. Thus, various labels may be employed, such as radioisotopes, biotin, fluorescers, chemiluminescers and the like. The choice of the label will be primarily directed to

convenience, sensitivity, minimizing background, minimizing interference with binding of the agonist to the MHC glycoprotein, and the like. Generally, the agonists will be at a concentration of about 0.1-50 times the concentration of the MHC glycoprotein.

A particularly preferred label is biotin. When biotin is used as "label," streptavidin which is in turn labeled with a wide variety of labels may be used for detection. Thus, the streptavidin may be labeled with radioisotopes, fluorescers, chemiluminescers, enzymes, colloidal particles, or the like. As indicated above, a variety of considerations will dictate which label will be employed.

The concentration of candidate moiety of interest will vary depending upon the concentration of agonist present in the medium, and the relative affinities of the candidate and agonist. Usually, the amount of the candidate will not differ by more than about 100-fold from the amount of agonist present in the medium.

According to the invention method, the various components (e.g., MHC glycoprotein, agonist and test compound) are combined and allowed to stand for sufficient time for the mixture to come to equilibrium. Generally the temperature is at about 37°C. Usually, the time for reaching equilibrium will be at least about 0.5 hours, more usually about 12 hours, and will generally not exceed about 48 hours. While a rate determination can be used, where a plurality of samples are employed and each sample analyzed for the amount of complex formation, it will usually be sufficient to do a single determination at varying concentrations of candidate.

Suitable candidate moieties include any small molecular weight material which is thought to block the MHC glycoprotein of interest. Typically, these

candidates are peptides of on the order of 5 or more amino acids or are small organic molecules which mimic the conformation of such peptides. A wide variety of candidates is generally known in the currently active field of rational drug design. There is no theoretical limitation on the range of candidates, and any putatively active compound may be employed.

In one strategy a soluble MHC glycoprotein in solution is used in the complex formation reaction. The labeled agonist complexed with the MHC glycoprotein is separated from free agonist and the amount of complexed agonist determined as a measure of the affinity of the candidate to the MHC glycoprotein. The method provides for a rapid, simple and accurate technique for screening large numbers of candidates and obtaining relevant values for the affinity of the candidate to MHC solubilized glycoproteins which is thought to correlate to the affinity found when said MHC glycoproteins are in their natural locations in cellular membranes.

In carrying out the method, a solution is prepared of the MHC glycoprotein, where the glycoprotein may be the naturally occurring dimeric glycoprotein freed of the cell membrane or a soluble glycoprotein which lacks the transmembrane region. The latter can be prepared in a variety of ways, using recombinant techniques, where the genes encoding the alpha and beta chains of a Class II MHC glycoprotein or the alpha chain of the Class I MHC glycoprotein may be truncated by removal of all or a portion of the transmembrane region. Conveniently, the transmembrane sequence may be replaced with a region capable of linking to a lipid. See, for example, Caras et al., Science (1987) 238:1280; Tykocinski et al., Proc Natl Acad Sci USA (1988) 85:3555. The lipid may then be removed by an appropriate esterase, e.g., phosphatidyl inositol-specific phospholipase C.

The concentration of the MHC glycoprotein will generally be in the range of about 0.01 to 50 μM, more usually about 0.1 to 1 μM. This range is convenient and is not critical, since in some experiments it may be desirable to use either higher or lower concentrations, depending upon the affinity of the mixtures to be bound, the concentration of moiety employed, and the like.

The medium will generally be buffered at about physiologic pH, pH 4.5-8, preferably about 5-6.5, with a buffer concentration of about 10 to 200 mM. Other additives may include salt, to a concentration of about 10 to 20 mM, or nonionic surfactants. The nonionic surfactants will generally be present in a concentration of about 0.1 to 2%. If a peptide agonist is used, the peptide will generally be at least about 3 amino acids and not more than about 30 amino acids, preferably being from about 3 to 16 amino acids, more preferably from about 5 to 15 amino acids.

Once sufficient MHC-agonist complex has been formed, the complex may be separated in a variety of ways. The complex may be separated from free agonist by gel filtration, gel electrophoresis in a nonreducing SDS polyacrylamide gel, or by binding the complex to a plate coated with antibodies or other affinity reagent (ligand) specific for the MHC glycoprotein. For gel fractionation, Sephadex G-50 is found to be useful. For gel electrophoresis, a 12.5% SDS-PAGE gel under nonreducing conditions is found to be satisfactory.

Preferably, however, affinity separation, particularly antibody separation is used, preferably on a plate, more particularly on a multi-well plate. For example, by employing excess antibody or fragment thereof specific for the constant region of one of the subunits of the MHC glycoprotein, one can capture the complex between the agonist and the MHC glycoprotein. One can

then quantitate the amount of agonist bound to the surface by washing away any nonspecifically bound or unbound labeled agonist and then detecting the agonist present bound to the surface. Washing may be performed with any convenient buffered medium, including the medium employed for complex formation. Where the agonist is labeled with biotin, by employing labeled avidin one can obtain a plurality of labels bound to a single agonist-MHC glycoprotein complex. Of particular interest with the avidin is the use of fluorescent labels, particularly lanthanide chelates, more particularly europium chelates, or enzymes, particularly horseradish peroxidase. These labels may be quantitated in accordance with conventional procedures, there being numerous fluorimeters for detecting fluorescence from lanthanide chelates and numerous spectrophotometers for detecting peroxidase substrates which result in chromophores.

In a second strategy, particularly useful for determination of the ability of a candidate to form a complex with an MHC glycoprotein present in a crude lysate, the MHC glycoprotein of interest is first captured on a solid support and the remaining components of the lysate washed free of the adsorbed MHC glycoprotein on the support. The coupled support is then treated with a reaction mixture containing the agonist capable of detection when complexed with the MHC glycoprotein and the competing candidate moiety. The nature of the labeling of the agonist, if needed, and of the concentrations of the competing substances in the reaction mixture is similar to that described above with respect to the use of solubilized MHC glycoprotein.

The derivatized solid support is prepared by passive adsorption or by covalent coupling of an affinity reagent specific for the MHC glycoprotein of interest by standard techniques well known in the art. Typically,

microtiter plates or other multiwell reaction matrices are used as solid support. This method has the advantage of removing contaminants from the crude lysate which might interfere with the binding of the candidate and/or agonist to the MHC glycoprotein. The activity of such contaminants must be minimized since binding of agonists to MHC glycoproteins occurs only at elevated temperatures where proteases and the like present in the lysates would exhibit activity.

Thus, in this method, a reaction mixture containing the relevant amounts of agonist and candidate is incubated for a suitable time period, usually about 3-48 hours, in the presence of the MHC complex coupled to solid support. At the end of the incubation time the solid support is removed from the reaction mixture, washed, and the agonist bound to MHC glycoprotein determined according to the nature of the label as described above.

In a third strategy, the first competition protocol may be used, but may be further optimized by preloading the MHC glycoprotein with a homogeneous, usually peptide agonist, which has a suitable off rate to permit the binding of the agonist/competitor combination. In this method, the purified MHC glycoprotein or the crude lysate is incubated with a preloading agonist, generally overnight in a suitable buffer, generally containing octylglucoside, for a time sufficient to replace the heterogeneous endogenous peptides contained in the native MHC glycoprotein with the preloading homogeneous moiety. The preloaded MHC complexes are then diluted and used in the assay systems described above. This approach provides a uniform provision of "empty" MHC glycoprotein binding domains for binding or coupling with the agonist or candidate. Generally, the acceleration of what is otherwise the rate-limiting step in the

association of the candidate or agonist provides a shorter assay time and provides a more accurate determination of the association rate and affinity of the candidate. By providing a preloading condition where it is unnecessary for the incoming agonist or candidate to dislodge the preloaded moiety, the affinities of the competitors for the unloaded MHC glycoprotein can be compared more readily. Because the MHC molecule is preloaded with a peptide with a defined dissociation rate, the assay can be run for a shorter period of time, preferably three hours.

In a fourth strategy, the MHC glycoprotein is preloaded with a homogeneous, labeled peptide agonist which has been demonstrated to be displaced in the presence of other peptides. In this method, the purified MHC glycoprotein is incubated with the labeled agonist, generally overnight in a suitable buffer, generally containing octylglucoside, for a time sufficient to replace the heterogeneous endogenous peptides with the preloading moiety. The preloaded MHC complexes are then diluted into a solution of a displacing candidate moiety for about 0.5 hour and loss of counts with and without the candidate moiety is monitored as described above.

An additional aspect of the invention obviates the necessity to label the agonist used in the competition assays. In this approach, T-cells which have been primed with the agonist are used to detect the presence of the agonist in the complex. In this method, the complex is used in a T-cell proliferation assay with the primed T-cells. Enhanced proliferation of the primed T-cells is a measure of the binding of the agonist to the complex. This method can also be used to detect any test moiety in the complex by priming the T-cells with test moiety.

The following examples are offered by way of illustration and not by way of limitation.

Example 1

Purification of HLA-DR and DQ Proteins

Anti-DR (LB3.1) and anti-DQ (IVD12) affinity columns were prepared using spherical cellulose resin (Amicon) and 40 mg of each of the antibodies. The maximum amount of solubilized Class II that combined to the columns is twice the molar concentration of the bound antibody. Therefore, 40 mg of immunoglobulin can bind at most 32 mg of Class II MHC glycoprotein. Practically, only between 10-30% of the theoretical capacity of an affinity column is allowed, which corresponds to between 3 and 9 mg of DR and DQ. A comparison of the L243 and LB3.1 columns was performed with an NP-40 detergent extract from 1.8 x 10^{10} cells which was equally divided, loaded on each column, and eluted with either 5 or 15 minute exposure to pH 11.5 buffer. The yields are as follows:

	5'exposure (mg)*	15'exposure (mg)	(mg)	total yield (mg)
L243	1.8	0.886	2.69	
LB3.1	2.10	2.02	4.12	6.81
IVD12	1.37	3.21	4.58	4.58

*measured by BCA assay using BSA as a standard.

Example 2

Separation of Radioactive Peptide-MHC Complex From Free Peptide by Gel Filtration

An iodinated HA 307-319 analog (2 μM) was incubated with HLA-DR4Dw4 (2 μM) overnight in PBS/1% octylglucoside (50 μL total volume). The reaction mixture was applied to a 25 ml Sephadex G-50 column. Fractions (0.5 ml) were eluted in PBS/1% octylglucoside and counted. The elution profile of the radiolabeled peptide was also determined. A peak was observed at from about 40 to 50 relative elution volume percent which could be attributed to the complex free of the peptide. This peak was not observed when the peptide control was performed.

Example 3

Separation of Bound From Free Peptide Using Antibody-Coated Plates

a) A radioactive iodinated analog of HA 307-319 at 1 μM was incubated overnight with DR4Dw4 at 2 μM in PBS/1% octylglycoside with or without HA 307-319 as a competitor in a volume of 50 μl. At the same time, microwell plates were coated with LB3.1 overnight at 4°C, washed with PBS/0.05% Tween/0.1% BSA and blocked with PBS/5% FCS at 4°C. Fifty μl of PBS/1% octylglucoside/5% FBS was then added to each well followed by 50 μl of the incubation mixture of peptide and Class II MHC glycoprotein. After an hour of incubation the plate was washed and counted.

b) An agonist corresponding to residues 307-319 of influenza hemagglutinin (HA 307-319), biotinylated at the amino terminus, was incubated with affinity-purified DR4Dw4 (2 μM) PBS/1% octylglucoside). Peptide DR complexes were separated from free peptide as with the iodinated HA except 25 μl of PBS/1% octylglucoside (5%

FCS) and 10 μl of the incubation mixture were added to the wells of the antibody-coated ELISA plates. After incubation for an hour the plate was washed with PBS/0.05% Tween/0.1% BSA and the amount of HA 307-319 bound was quantitated by incubation with ^{125}I streptavidin (6-30 mg/well) for an hour at 4°C followed by washing and counting.

c) Competition for binding of biotinylated HA 307-319 to DR4Dw4 was performed as in b) with or without several HA monosubstituted peptides (e.g., lys, ser or phe at position 309) (200 μM) in PBS/1% octylglucoside. With antibody LB3.1, the counts observed in the presence of DR antigen as compared to background were about twice that of background, while in the presence of a nonradioactive competitor, the counts were somewhat lower than background. With antibody IVD12, the observed counts in the presence of DR were lower than background. Typical results for inhibition curves are shown in Figure 1.

For experiment b), except for the combination of the radioactive peptide and the DR antigen, substantially no signal was observed.

In the competition experiment c), with peptide competitors corresponding to analogs containing point substitutions (i.e., lysine or serine at 309), the counts were about the same as background, while in the presence of nonradioactive peptide 307-319 or phenylalanine at position 309 there was substantially no radioactivity observed.

Example 4

Comparison of Nonradioactive and Radioactive Detection Systems for the Binding of a Biotinylated Hemagglutinin Analog to DR4Dw4

The assay was formed as described in Example 3b using a biotinylated alanine backbone analog of HA (AAFKAAEAAAARA) at 2 μM and DR4Dw4 at 0.5 μM. The peptide DR complexes were quantitated using either a fluorescent europium-conjugated streptavidin, a horseradish peroxidase-conjugated streptavidin or ^{125}I-conjugated streptavidin. Each staining reagent was titrated to obtain the maximum signal over background possible.

With streptavidin labeled with the fluorescent europium chelate, signal-to-noise ratios as great as seen with ^{125}I streptavidin (between 80-100:1) were observed. Good signal-to-noise ratios (10-20:1) were also observed with horseradish peroxidase-streptavidin conjugates.

Example 5

Assay of Specific Binding in a Crude Lysate

The monoclonal antibody LB3.1, specific for DR Class II molecules at 2 μg/ml, 200 μl/well, was coated onto a Costar EIA-RIA plate in 50 mM Tris-HCl, pH 9.0, either overnight at 4°C or for 1 hour at 37°C. The plate was washed and blocked for 1 hour at room temperature with 5% FCS/PBS and then washed 3-4 times with 0.05% Tween 20/0.01% azide/PBS (wash buffer) using a Titertek plate washer. Cell lysates assessed to contain approximately 20 nM DR MHC glycoprotein was then incubated for 4 hours at 4°C on the coated plate. The plate was washed 3-4 times with 0.05% Tween 20/0.01% azide/PBS. Biotinylated HA peptide 307-319 was then added to the plate in 5% FCS/1% octylglucoside (OG)/PBS

at 200 µl/well and incubated overnight at 37°C in a CO_2 incubator.

The plate was washed 3-4 times with wash buffer and incubated for 4 hours at 4°C with 200 µl of europium chelated streptavidin (Pharmacia/LKB Nuclear) at 60 ng/ml. After an additional washing cycle, the plate was treated for 30 min at room temperature with 200 µl/well of enhancement solution (Pharmacia/LKB) which releases bound europium for detection in a 1234 Delfia research fluorometer (Pharmacia/LKB).

Typical results are shown in Figure 2. Binding curves proportional to concentration of the biotinylated HA 307-319 peptide are obtained when a Priess cell lysate was used (solid circles) as well as when purified DR4 MHC glycoprotein at 10 nM (open circles) or the lysate from Cos 7 cells transfected with DR4Dw4 (open squares) were used to provide the MHC glycoprotein. Mock transfected Cos cells used as a control (closed squares) showed no uptake of the labeled peptide.

Example 6

Alternate Plate Assay Protocol

The assay was conducted in a manner similar to that set forth in Example 5 except that the antibody-coupled plates were prepared as follows:

Avid-HZ™ plates (Bioprobe International) which covalently couple oxidized antibody to their surfaces were used. LB3.1 monoclonal antibody was first oxidized by diluting to 10 µg/ml in 50 mM acetate buffer pH 5, followed by addition of a 1/10 volume freshly prepared 10 mM sodium metaperiodate. After 30 minutes at room temperature, the reaction was stopped by addition of 1/10 volume of 20 mM ethylene glycol in acetate buffer. 115 µl of the oxidized antibody solution were added to the wells of an Avid-HZ™ plate and incubated overnight at

4°C. The plates were then washed 4 times with wash solution (PBS/0.05% Tween 20/0.01% sodium azide) and then blocked for 1 hour at 4°C with PBS/5% FCS/0.01% sodium azide.

125 µl of 20 nm DR4Dw4 in PBS/0.75% octylglucoside/0.01% sodium azide (binding buffer) were added to each well and incubated for 4 hours at 4°C. The plates were washed 4 times with wash buffer and treated with 125 µl of biotinylated HA 307-319 peptide contained in binding buffer and incubated overnight at 37°c. The plates were then washed 4 times with wash buffer and incubated overnight at 4°C with 125 µl of europium chelated streptavidin (Pharmacia/LKB) at 60 ng/ml. After an additional wash cycle, the plates were treated for 1 hour at room temperature with 125 µl/well enhancement solution (Pharmacia/LKB) and read in a 1234 Delfia research fluorometer.

Results similar to those in Example 5 were obtained.

Example 7

Use of Preloaded MHC Glycoprotein

To determine a suitable preloading peptide, off rates were determined for several candidate preloading peptides: rat myelin basic protein 90-102 (RMBP90-102) which has the sequence HFFKNIVTPRTPA; HA derived protein 3.2 (HADP 3.2) which has the sequence RRFAAAYAARAAA; and HA derived protein 3.6 (HADP 3.6) which has the sequence RRFAAQYAARAAA. 50 nM of labeled preloading peptide was incubated for 48 hours with 400 nM DR4Dw4 in PBS, pH 7.0 binding buffer. Off rates were determined by diluting the complexes 1:40 into PBS and various concentrations of unlabeled HA 309-319 at various times, followed by capturing the complex on antibody plates coupled to LB3.1 as described above. As shown in Figures 3A-3C, none of

the tested peptides showed off rates that were affected by the presence of HA 307-319 and the dissociation rate for RMBP 90-102 was the most rapid of the three. Therefore, preloading with RMBP 90-102 was selected for use in the assays.

In the assay, 2 μM purified DR4Dw4 and 1 μM RMBP 90-102 were incubated overnight in PBS/0.75% octylglucoside/0.01% sodium azide binding buffer as described above.

Antibody capture plates were prepared using 115 μl LB3.1 at 2 μg/ml overnight at 4°C and washed and blocked as described above.

The preloaded complexes (i.e., DR4Dw4/RMBP 90-102) were diluted 1:100 and incubated with varying concentrations of biotinylated RMBP 90-102 or with 8.8 nM biotinylated RMBP 90-102 in the presence of varying amounts of the same peptide unlabeled. Controls using diluted nonpreloaded DR MHC glycoprotein were also run.

Aliquots (50 μl) of the reaction mixtures were removed to the capture plate and incubated at 4°C for 4-24 hours. The plates were washed as described above and treated with 125 μl europium-streptavidin at 60 ng/ml in assay buffer (calcium and magnesium-free PBS + 0.5% BSA, 20 μM diethylenetriaminepentacetic acid (DTPA) in 0.1% sodium azide) and incubated 2-24 hours at 4°C. After additional washing, the plates were enhanced and read as described above.

Typical results are shown in Figures 4A and 4B. Figure 4A shows a binding curve for biotinylated RMBP for control and preloaded DR. Figure 4B shows the percent inhibition obtained with varying concentrations of unlabeled RMBP. As shown above, the results are comparable for preloaded and nonpreloaded DR MHC glycoprotein. After titrating reagents, it has been determined that the screen will be run by preloading 2 μM

DR4Dw4 with 250 nM RMBP 90-102 by incubating in calcium, magnesium free PBS adjusted to pH 6.5 with 0.1 M KH_2PO_4 overnight at 37°C. The preformed complexes will be diluted 1/40 and reacted with the labeled agonist and the test compounds for 3 hours. The complexes will then be captured as described above.

Example 8

Detection of Ligand/Receptor Dissociation Rates

Ligand/receptor dissociation rates should be first order, depending only on the concentration of the complex present. In studying class II/peptide dissociation rates, rates are first order in some cases but not all.

To determine the dissociation rates, 400-500 nM DR4Ew4 was incubated with 50 nM biotinylated RMBP or 25 μm biotinylated HSP (19 kD heat shock protein 3-14 form Mycobacterium tuberculosis) overnight at 37°C as described previously. These preformed complexes were then diluted 100-fold into buffer with or without varying concentrations of HA 307-319. As can be seen in Figure 5A, the dissociation rate of RMBP is not affected by the second peptide. However, as seen in Figure 5B, the dissociation of HSP was accelerated by the presence of the other peptide. Since the dissociation rate increases with the concentration of the other peptide, this is clearly not a first order reaction.

This suggested a displacement model, where the second peptide pushes off the first peptide. Therefore, in a second preloaded screening assay, 800 nM DR4Dw4 and 6 μM biotinylated HSP 3-14 were incubated as described above. Complexes were diluted 40-fold into a buffer solution with or without the competitor candidate moiety and the displacement monitored by a decrease in counts at thirty minutes. Figure 6 shows displacement by peptide

competitor (RMBP), but any small molecule could potentially have the same effect.

Example 9

Detection by Sensitized T-Cell Proliferation

T-cell clones responsive specifically to HA 307-319 were prepared from an individual with HLA Class II phenotype DR4Dw4, DR7, DRw53, DQw8, DQw9 (as described in Example 1). Briefly, peripheral blood mononuclear cells were primed in vitro with 1/100 dilution of influenza virus vaccine (Parke Davis) in RPMI supplemented with streptomycin (100 μg/ml) penicillin (100 U/ml) and 5% pooled AB serum (Whittaker, Fredricksburg, MD). After incubation at 37°C in 5% CO_2 for 5 days, T-cell blasts were cloned by limiting dilution at 1 cell per 3 wells in the presence of a feeder mixture consisting of 10^6 allogeneic PBMC per ml (30 Gy irradiated), 10^5 cells of autologous EBV transformed B cells (50 Gy irradiated), 1/100 dilution of influenza virus vaccine and 1 μg/ml leucoagglutinin-A (Pharmacia). The suspension was plated in 96 well flat-bottom microtiter plates and incubated as described above. Growing cultures were transferred to a 24-well tissue culture plate and restimulated with the same feeder mixture. Three days later, 10% IL-2 was added and the cells were frozen or further expanded by restimulation. Specificity was determined with HA 307-319.

DR4Dw4 glycoprotein was affinity purified from Priess EBV-B cells as described in Example 1. Briefly, the cells were grown in RPMI medium supplemented with fetal calf serum and collected by centrifugation, washed with PBS and lysed with 1% Nonidet P-40. The cellular debris was removed by centrifugation and the lysate

loaded directly to a Sepharose CL-4B column connected in series with a monoclonal antibody LB3.1 cellulose column prepared by coupling 40 mg of antibody to 10 ml Matrex Cellufine Formyl (Amicon) according to the manufacturer's instructions. The columns were washed with 20 volumes of 10 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.1% deoxycholate. The LB3.1 cellulose column was then washed with 5 column volumes of 10 mM Tris HCl, pH 7.5, 1% OG and eluted with 50 mM glycine, pH 11.5, 1% OG. The fractions were adjusted to pH 7.5 with 2 M glycine, pH 2. The full fractions were dialyzed against 10 mM Tris HCl, pH 7.5, 1% OG and stored at 4°C.

To prepare the MHC glycoprotein/peptide complex, purified DR4Dw4 was incubated overnight at 37°C with HA 307-319 at varying concentrations in 1% OG/PBS. 50 µl of the mixture were added to 96-well flat-bottom ELISA plates which had been coated with LB3.1 by incubation overnight at 4°C in 5 µg/ml LB3.1 in 50 mM Tris HCl, pH 9. The mixture was incubated with the coated plates for 6 hours at 4°C and then washed twice with PBS and once with complete medium.

For T-cell proliferation assays, 3×10^4 T-cells were added in 200 µl of complete medium to each well (the T-cells were used 10-11 days after restimulation). After incubation for 24 hours, 1.0 µCi per well of tritiated thymidine were added, and the plates were incubated for another 18 hours. The samples were harvested on glass fiber filters using a semiautomatic harvester and thymidine incorporation was assessed by counting in a scintillation counter. The results of triplicate determinations are shown in Figure 7.

As shown in Figure 7, T-cell proliferation can be used to determine a binding curve for the test HA 307-319 peptide.

It is evident from the above results that rapid, efficient assays are provided for screening candidate moieties for binding affinity to particular MHC glycoproteins. Thus, the methodology allows for evaluation of a wide variety of candidates and their ability to interact with MHC glycoprotein and ultimately to modulate the immune response in a host having such MHC glycoprotein.

All publications and patent applications mentioned in the specification are herein incorporated by reference to the same extent as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

Claims

1. A method for determining the affinity of a test compound to an MHC glycoprotein, which method comprises:

combining, in a reaction mixture, cell-free dispersed MHC glycoprotein, an agonist capable of binding to said MHC glycoprotein to form a complex and capable of being detected when in said complex, and said test compound

under conditions wherein the test compound and the agonist compete for binding to the MHC glycoprotein;

separating MHC glycoprotein-bound agonist from unbound agonist; and

detecting the amount of agonist bound in the complex as a function of the concentration of test compound in the reaction mixture.

2. The method of claim 1, wherein the MHC glycoprotein lacks a complete transmembrane sequence.

3. The method of claim 1, wherein said separating is by means of gel filtration.

4. The method of claim 1, wherein the reaction mixture is at a pH in the range of about 4.5 to 8 and comprises from about 0.1 to 2% of a nonionic surfactant.

5. The method of claim 1, wherein said agonist is coupled to biotin and said detecting is by reacting said MHC complex with streptavidin containing label.

6. The method of claim 5, wherein said label is a fluorescent, radioactive, or enzyme label.

7. The method of claim 1, wherein said separating is by means of coupling said complex to a solid support wherein said solid support is coupled to an affinity ligand for said MHC complex.

8. The method of claim 7, wherein said affinity ligand is an antibody or immunologically reactive fragment thereof specific for MHC glycoprotein.

9. The method of claim 7, wherein said agonist is coupled to biotin and said detecting is by reacting said MHC complex with streptavidin containing label.

10. A method to determine the affinity of a test compound for a specific MHC glycoprotein, which method comprises treating a solid support to which the MHC glycoprotein is coupled with a reaction mixture containing an agonist capable of binding to the MHC glycoprotein to form a complex and capable of being detected when in said complex and said test compound, under conditions wherein the test compound and agonist compete for binding to the MHC glycoprotein;

removing said reaction mixture from the solid support; and

detecting the amount of agonist bound to the solid support as a function of the concentration of the test compound in the reaction mixture.

11. The method of claim 10, wherein said solid support has been modified to contain an affinity ligand for MHC glycoprotein.

12. The method of claim 11, wherein said affinity ligand is an antibody or immunologically reactive fragment thereof specific for MHC glycoprotein.

13. The method of claim 10, wherein said agonist is coupled to biotin and said detecting is by reacting said MHC complex with streptavidin containing label.

14. The method of claim 13, wherein said label is a fluorescent, radioactive, or enzyme label.

15. A method for determining the affinity of a test compound to an MHC glycoprotein, which method comprises treating said MHC glycoprotein preloaded with a first agonist with a reaction mixture containing the test compound and a second agonist capable of binding to said MHC glycoprotein to form a complex and capable of being detected when in said complex under conditions wherein the test compound and second agonist compete for binding to said MHC glycoprotein;

wherein said MHC glycoprotein is preloaded with a first preloading agonist; and

detecting the amount of second agonist bound in the complex as a function of the concentration of test compound in the reaction mixture.

16. The method of claim 15, wherein said second agonist is coupled to biotin and said detecting is by reacting said MHC complex with streptavidin containing label.

17. The method of claim 16, wherein said label is a fluorescent, radioactive, or enzyme label.

18. The method of claim 15, wherein said preloaded MHC glycoprotein is diluted prior to adding said reaction mixture to said preloaded glycoprotein.

19. A method for determining the affinity of a test compound to an MHC glycoprotein, which method comprises preloading said MHC glycoprotein with a displaceable, labeled agonist adding the test compound and detecting the amount of labeled agonist displaced by the test compound.

20. A method to determine the presence or level of a moiety contained in an MHC glycoprotein complex with said moiety which method comprises contacting said complex with T-cells primed with said moiety and determining the extent of proliferation of said T-cells.

21. The method of claim 1, wherein said detecting is by contacting said complex with T-cells primed with the agonist and determining the extent of proliferation of said T-cells.

22. The method of claim 10, wherein said detecting is by contacting said complex with T-cells primed with the agonist and determining the extent of proliferation of said T-cells.

23. The method of claim 15, wherein said detecting is by contacting said complex with T-cells primed with the second agonist and determining the extent of proliferation of said T-cells.

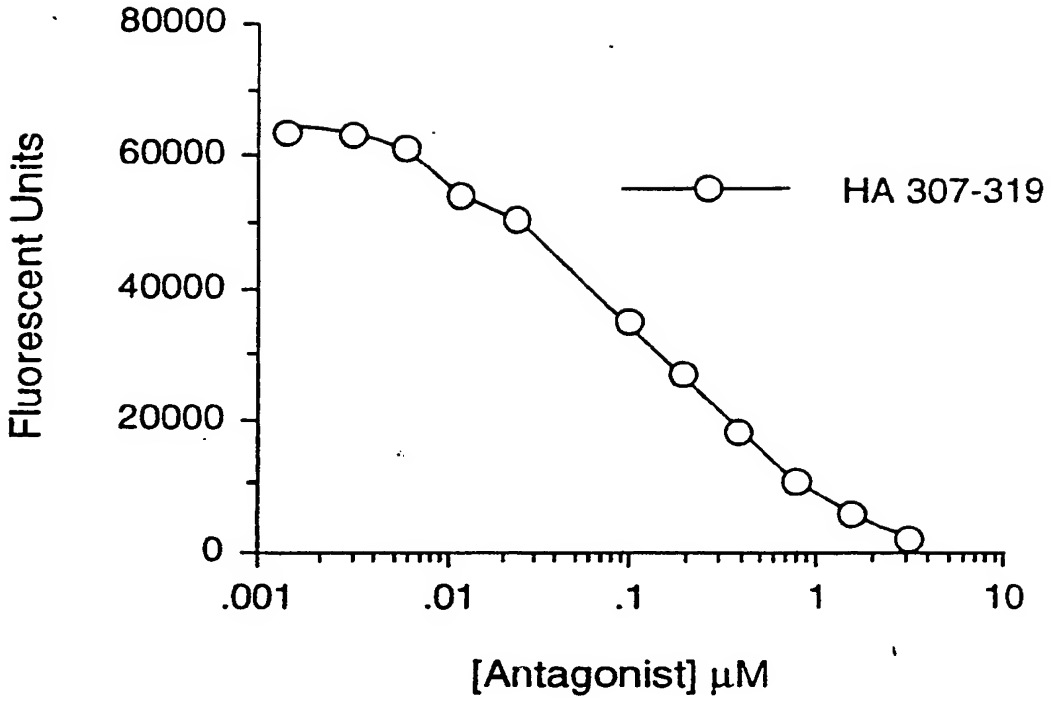

Fig. 1

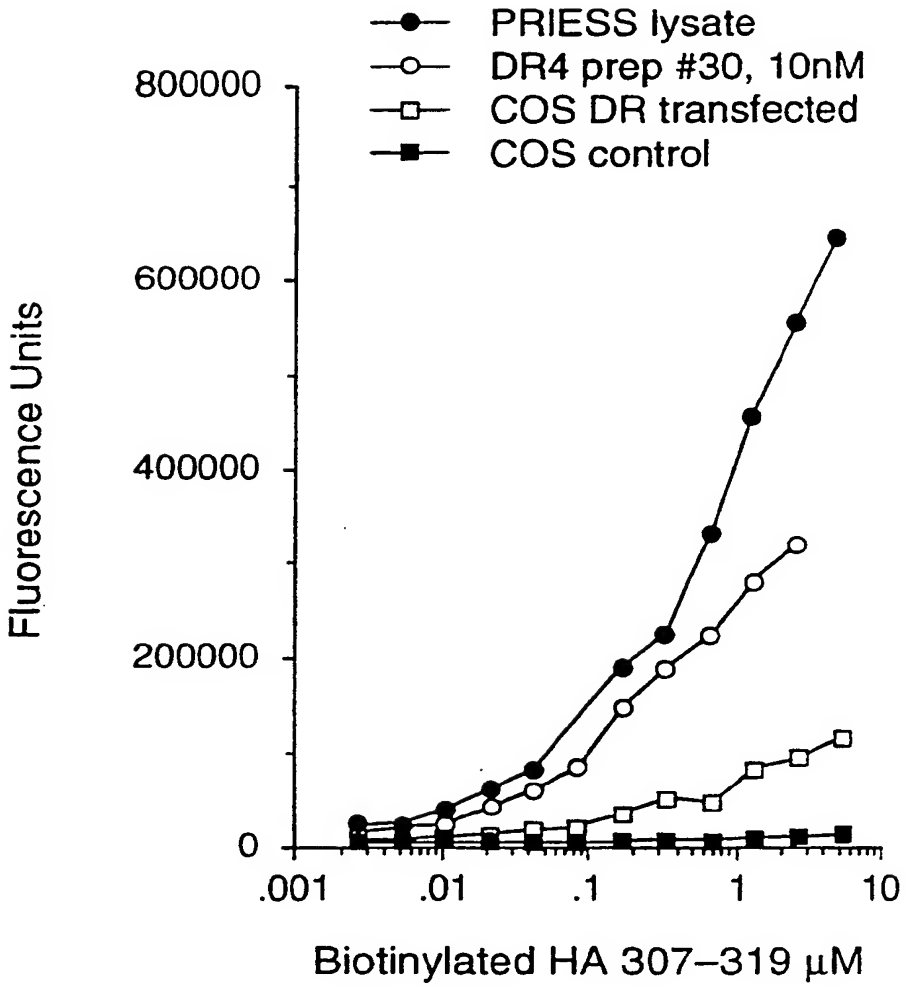

Fig. 2

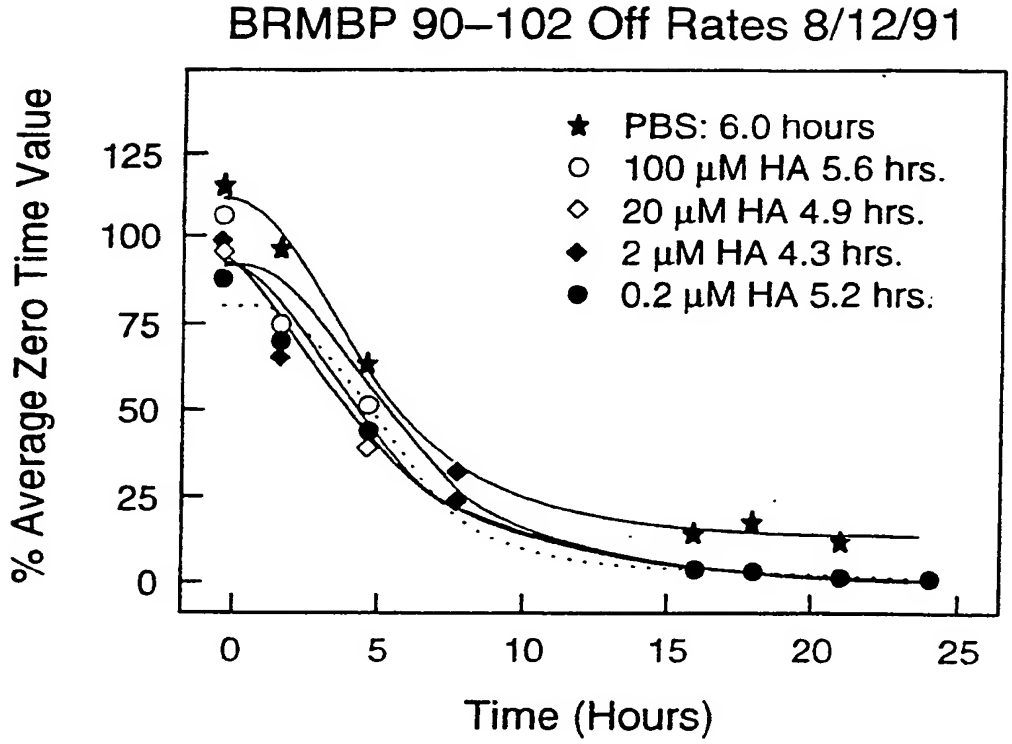

Fig. 3A

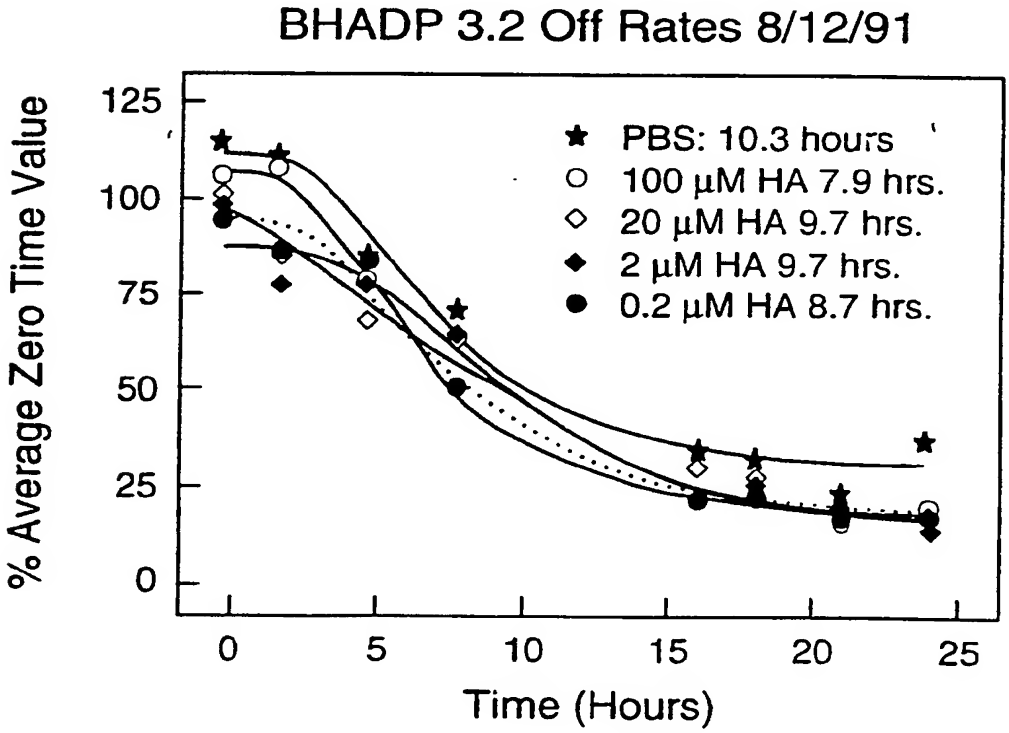

Fig. 3B

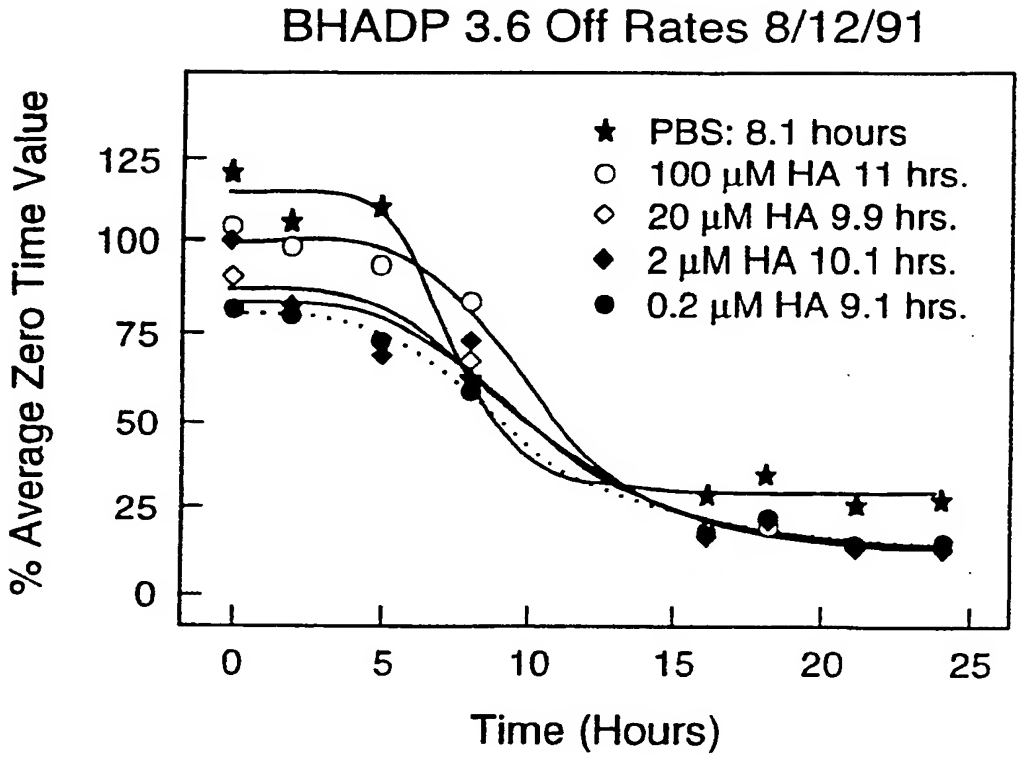

Fig. 3C

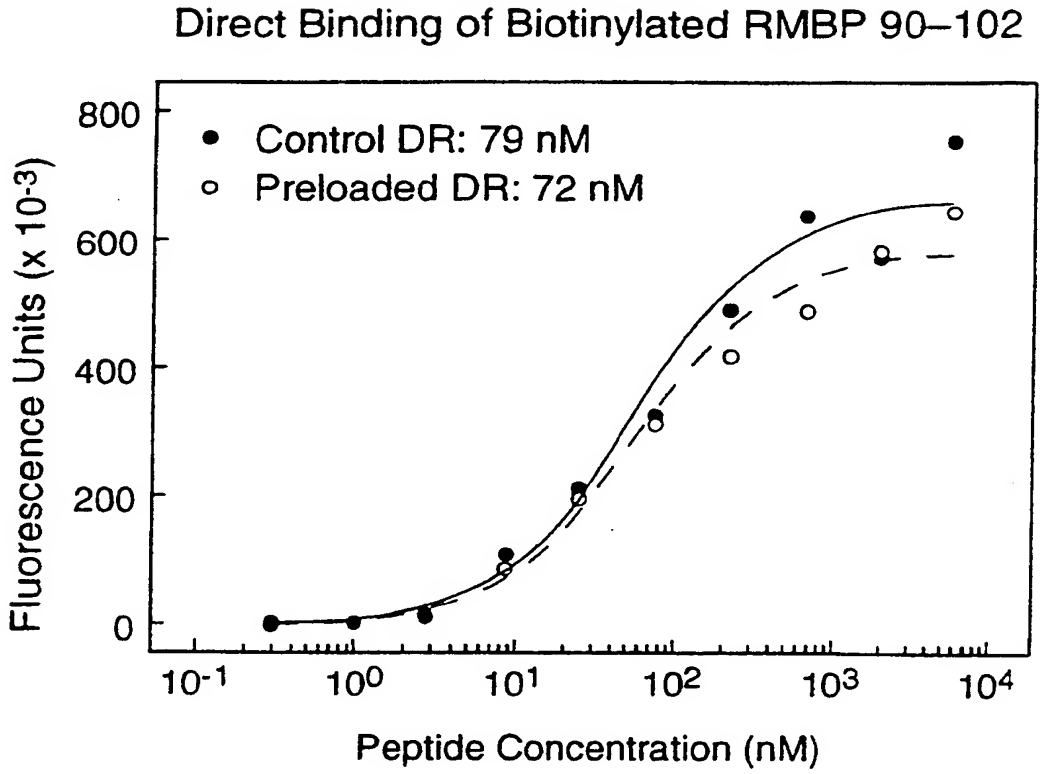

Fig. 4A

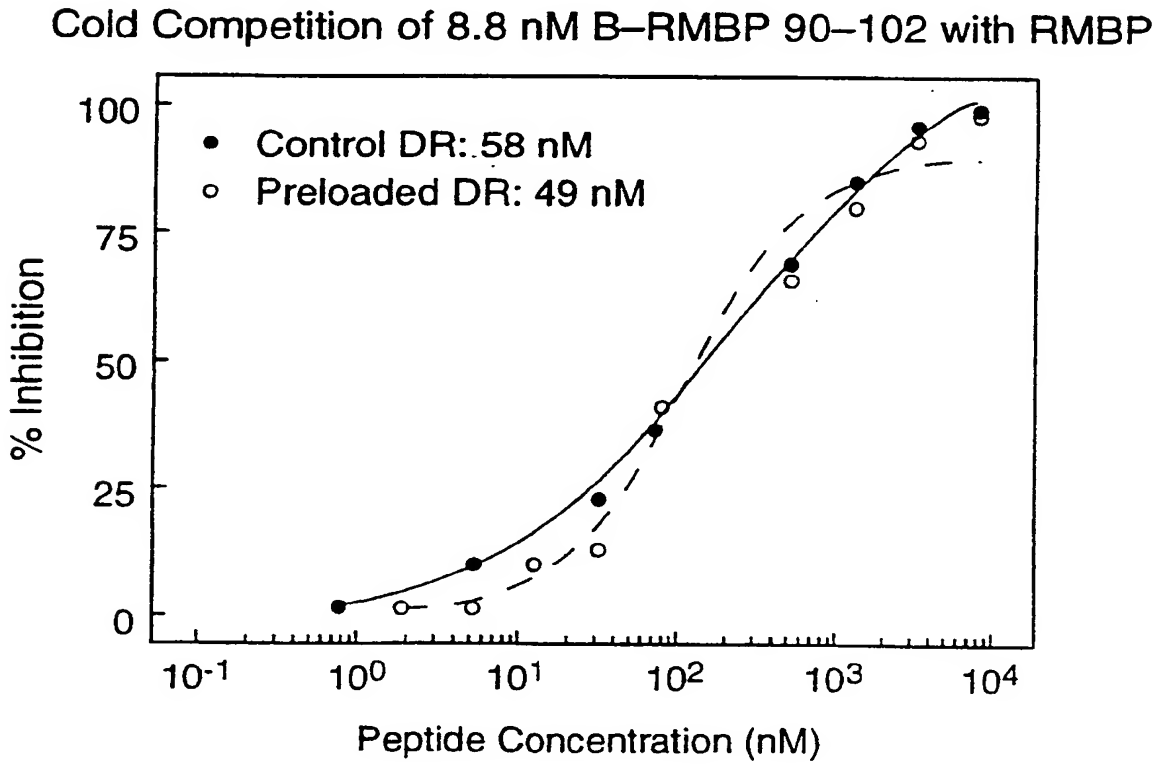

Fig. 4B

SUBSTITUTE SHEET

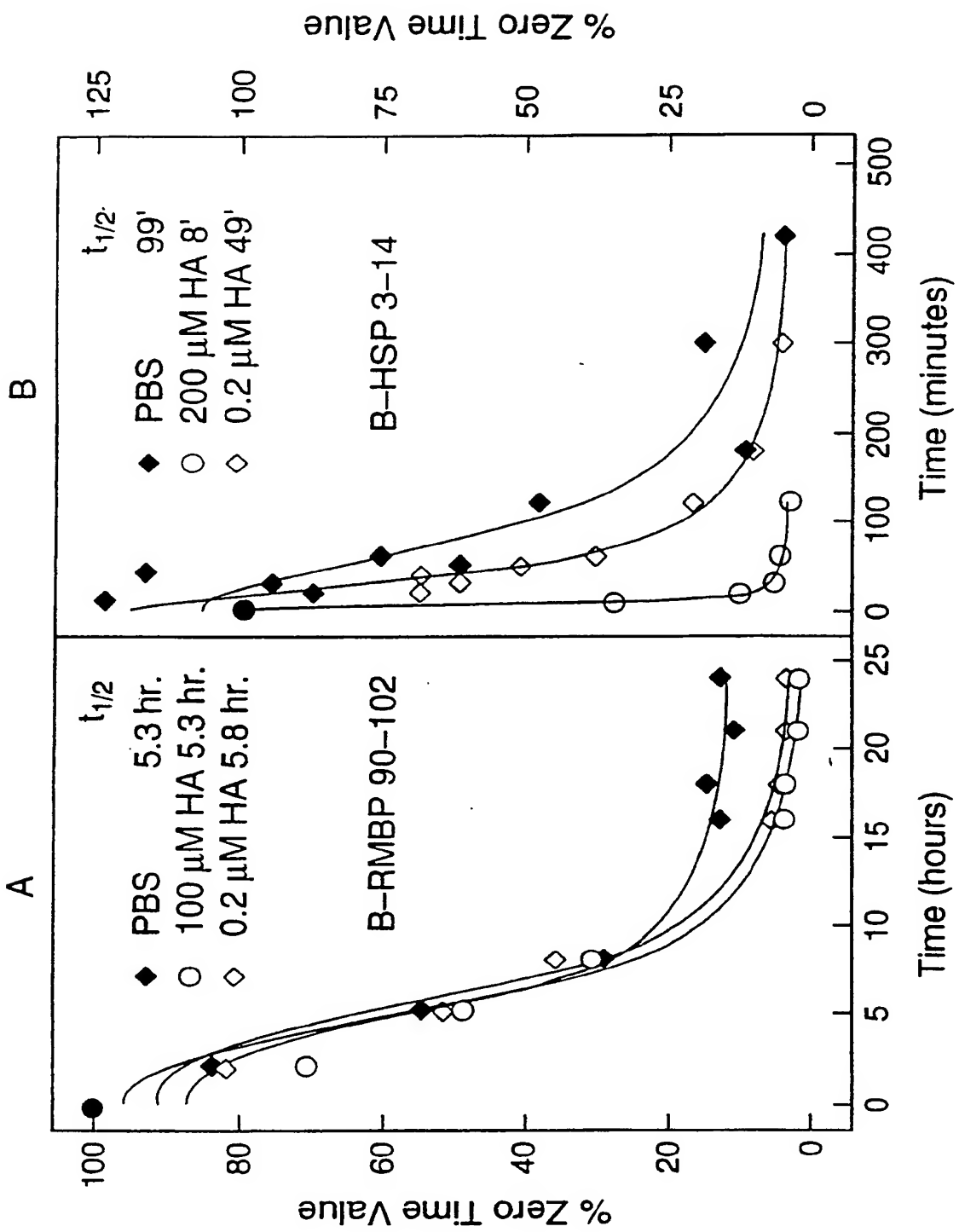

Fig. 5

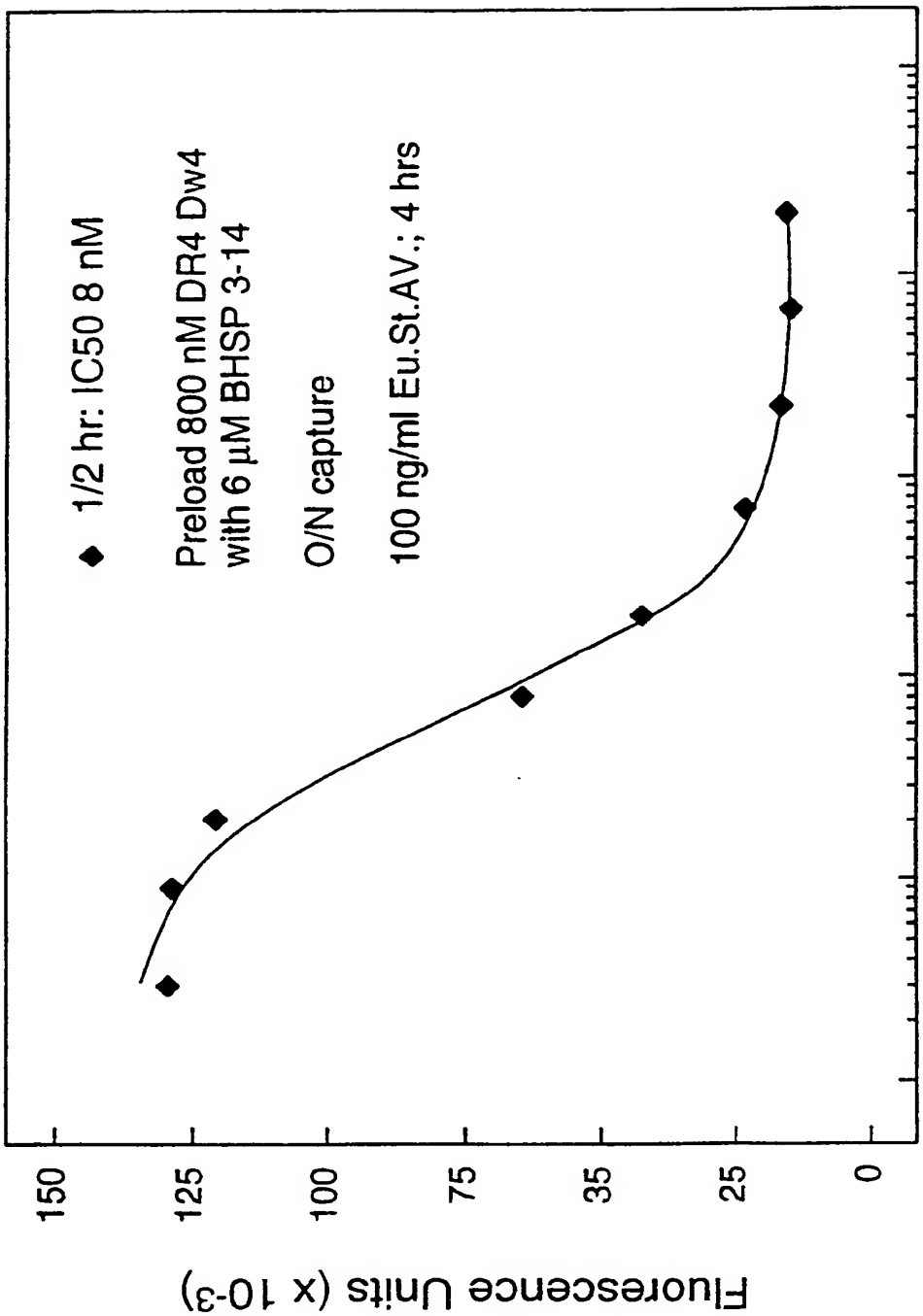

Fig. 6

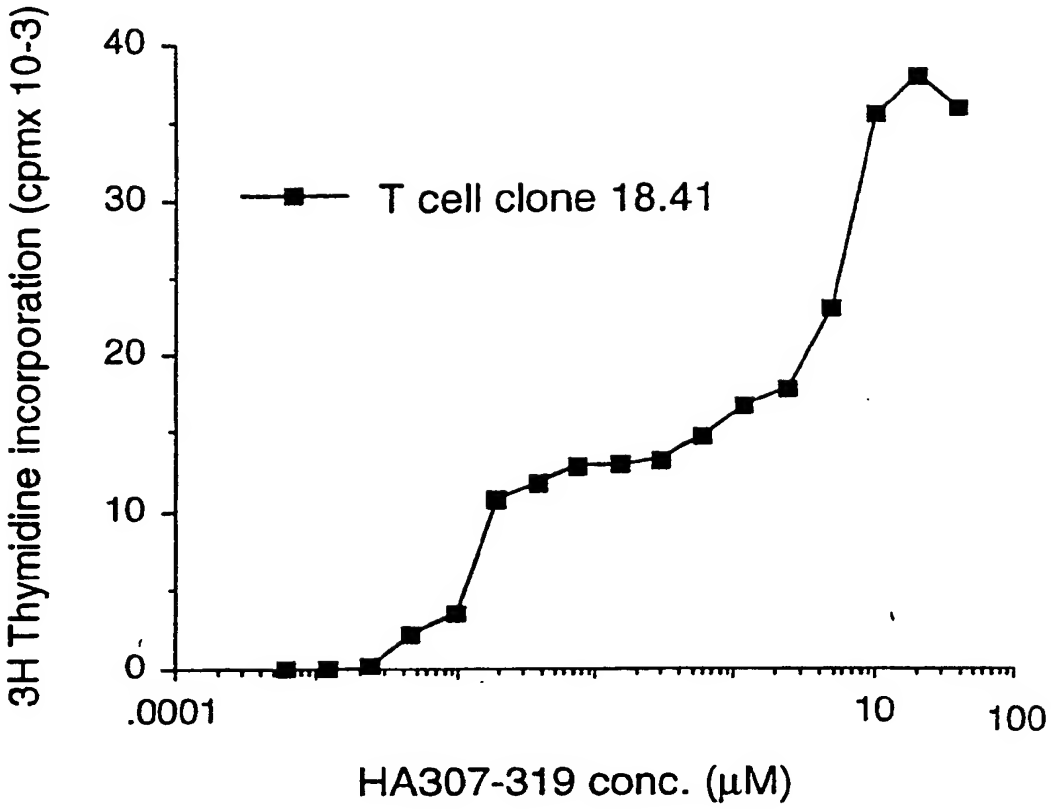

Fig. 7

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/08080

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/02; G01N 33/538, 33/543, 33/563, 33/566, 33/567

U.S.Cl.: 435/7.21, 7.24, 7.5, 7.8, 7.9, 7.92, 29; 436/501, 503, 512, 518, 541

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.Cl.:	435/7.21, 7.24, 7.5, 7.8, 7.9, 7.92, 29; 436/501, 503, 512, 518, 541

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Science, vol. 235, issued 20 February 1987, J.L. Marx, "Histocompatibility restriction explained", pages 843-844. See page 844, col. 3, last paragraph.	1-14 and 19
Y	Nature, vol. 328, issued 30 July 1987, A. SETTE ET AL., "Structural characteristics of an antigen required for its interaction with Ia and recognition by T-cells", pages 395-399. See legend to Table I.	1-14 and 19
Y	Science, vol. 235, issued 13 March 1987, S. Buus et al., "The relation between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunogenic peptides", pages 1353-1358. See legend to Table 2, para. spanning cols. 1-2 of page 1354 and para. spanning pages 1354-1355.	1-14 and 19
Y	US, A, 4,048,298 (NISWENDER) 13 September 1977, see col. 1, lines 44-65 and col. 2, lines 1-9.	7-9
	Continued on extra sheet.	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11 February 1992	27FEB 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	David A. Saunders roz

Form PCT/ISA/210 (second sheet) (Rev. 11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US, A, 4,120,945 (GUTCHO ET AL) 17 October 1978. See Abstract.	19
Y	US, A, 4,228,237 (HEVEY ET AL) 14 October 1980. See col. 1, line 67—col. 2, line 15 and col. 3, line 40—col. 4, line 5.	5-6, 9 and 13-14
Y	US, A, 4,478,946 (VAN DER MERWE ET AL) 23 October 1984. See col. 17, lines 23-47.	10-14